Curr Genet (1986) 10:557–560

Current Genetics

Isolation of mutants sensitive to 2-aminopurine and alkylating agents and evidence for the role of DNA methylation in *Penicillium chrysogenum*

S. D. Rogers*, M. E. Rogers, G. Saunders, and G. Holt*

School of Biotechnology, Faculty of Engineering and Science, The Polytechnic of Central London, 115 New Cavendish Street, London W1M 8JS, UK

Summary. Using high performance liquid chromatography, the presence of N^6-methyladenine has been found at a level of 0.1 mol percent in DNA extracted from *Penicillium chysogenum*. No 5-methylcytosine was detected. A mutant strain HP547, which is sensitive to the lethal effects of N-methyl-N′-nitro-N-nitrosoguanidine, methylmethane sulphonate and the base analogue 2-aminopurine shows an increased spontaneous mutation rate and no detectable DNA methylation. Comparison of restriction enzyme digests of wild type and undermethylated strains indicated that methylation was occurring at a different sequence to that of the Dam methylase system of *E. coli*.

Key words: Methylation – DNA – *Penicillium*

Introduction

The presence of methylated bases in the DNA of many prokaryotes and eukaryotes is thought to reflect a role in numerous cell processes. For example in *Escherichia coli*, where both 5-methylcytosine (5-MeC) and N^6-methyladenine (N^6-MeA) occur, DNA methylation is thought to be involved in restriction modification systems (Smith et al. 1972; Hattman et al. 1972). All studied higher eukaryotes contain 5-MeC as a minor base in their DNA, which may be a factor controlling gene expression at the level of transcription (Adams and Burdon 1982; Ehrlich and Wang 1981). Analyses of methylated base content of DNA from lower eukaryotes and in particular fungi, have been limited. Although Hattman et al. (1978) initially reported the detection of 5-MeC in *Saccharomyces cerevisiae*, subsequent publications indicate that 5-MeC may not be present (Adams and Burdon 1982). That patterns of DNA methylation are not universal amongst lower eukaryotes, is indicated by the findings that cytosine is unmodified in *Aspergillus niger* (Tamane et al. 1983), whereas the slime mould *Physarum polycephalum* contains significant amounts of 5-Mec (Whittacker and Hardman 1980). In the present study the base content of *Penicillium chrysogenum* was analysed using high-performance liquid chromatography (HPLC) and N^6-MeA but not 5-MeC found to be present.

DNA methylation in *P. chrysogenum* is of particular interest because of its possible involvement in mismatch repair and base analogue mutagenesis. The DNA of *E. coli* contains 0.5 mol percent N^6-MeA and Dam mutants, defective in DNA-adenine: S-adenosylmethionine methyltransferase activity, have been shown to be undermethylated (Marinus and Morris 1973). Strains of *E. coli* bearing *dam* mutations are also defective in the repair of mispairing lesions caused by base analogues (Glickman et al. 1978). Directed correction of mismatches occurring at replication has been proposed to occur in *E. coli* via a system which involves recognition of newly snythesized undermethylated DNA (Glickman and Radman 1980). As part of a general study of DNA repair mechanisms and mutagenesis in *P. chrysogenum* (Holt et al. 1984), strains showing the *E. coli* Dam phenotype have been investigated as well as revertants/suppressors obtained from these mutants. The results are discussed in relationship to evidence for a mismatch repair mechanism in *P. chrysogenum* with similarities to that of bacterial systems.

* *Present address:* The Biological Laboratory, The University of Kent at Canterbury, Canterbury, Kent CT2 7NJ, UK
Offprint requests to: S. D. Rogers

Materials and methods

Strains. The strains of *Penicillium chrysogenum* used in this work were derivatives of the wild type isolate NRRL 1951 (Raper and Alexander 1945). *P. chrysogenum* strains were given the prefix code HP. The progenitor strain HP36, from which all mutagen sensitive strains were isolated was a double auxotroph requiring aneurine (*an*) and a reduced nitrogen source (*nia*). Strains isolated on the basis of cross-sensitivity to 2AP and MNNG were designated Aps, carrying an *aps* mutation. Revertants obtained showing wildtype sensitivity to 2AP were likely to be due to suppressor mutations (*sup*). The genotypes of the strains used in this study were as follows: HP36 – *an-004, nia-003;* HP547 – *an-004, nia-003, apsA-001;* HP548 – *an-004, nia-003, aps 002;* HP549 – *an-004, nia-003, (apsA001 supA001);* HP550 – *an-004, nia-003 (aps-002 sup-002).*

Media and culture conditions. P. chrysogenum was maintained on slants of Aspergillus complete medium (ACM). Isolation of mutants and mutagenic studies were performed using Aspergillus minimal medium (AMM), supplemented with aneurine, 0.85 μg/ml and ammonium tartrate 0.32 mg/ml. Liquid culture was performed in liquid ACM. For AMM and ACM, see Ditchburn et al. (1974). Incubation was at 26 °C throughout.

Mutagen treatments and isolation of 2AP and MNNG sensitive strains. The protocol for the use of MNNG has been described previously (Rogers and Holt 1984). 2AP survival curves were constructed by incorporation of 2AP into agar at concentrations of 50, 100, 250 and 500 μg/ml. Where sensitivity to a mutagen was determined by a plate test, a disc method (see Rogers and Holt 1984) was employed. 20 μl of each mutagen were added to a disc at the following concentrations: MNNG, 50 μg/ml; 2AP, 10 mg/ml in dimethylsulphoxide (DMSO); methylmethane sulphonate (MMS), 0.002% (v/v). Where a mutagen was dissolved in DMSO, a suitable control plate test was performed. Strains HP547 and HP548 were isolated from HP36 following treatment of a spore suspension with MNNG for 20 min to give approximately 10% survivors. The resulting surviving colonies were screened for 2AP and MNNG sensitivity in a similar manner to that used previously to isolate 4-nitroquinoline-1-oxide (4NQO) sensitive strains (see Rogers and Holt 1984). Spontaneous revertants from HP547 were obtained by spreading 1×10^6 spores onto AMM containing 2.5 mg/ml.

Forward mutation assays. Spontaneous and induced mutation rates were measured by forward mutation to resistance to candidine and 5-fluorouracil (5FU) employing an expression period as described by Rogers and Holt (1984). Levels of mutagens for induced mutation were as for survival curve construction. 2AP was incorporated into the lower layer of agar for the candidine system. When using the 5FU method, spores were spread onto cellophane on agar containing various concentrations of 2AP. After 6 h incubation at 26 °C, cellophanes were transferred to 5FU agar.

Extraction and hydrolysis of DNA. All chemicals used were of Analar grade. Large quantities of DNA were extracted by the method of Saunders et al. (1984). Approximately 1mg DNA from each strain was required for analysis. RNA was separated from DNA by passage through a Sephacryl S-1000 (Pharmacia, Sweden) column (60 cm x 2.5 cm). Elution was with 10 mM Tris, 1 mM EDTA, pH 8.0 and the flow rate 0.15 ml/min. 3 ml fractions were collected in a Uniscil UFC 120 fraction collector. DNA purity was checked by agarose gel electrophoresis. Fractions containing high molecular weight DNA were pooled, precipitated with ethanol and dissolved in 2 ml of distilled water. Approximately 500 μg DNA were precipitated and following centrifugation the resulting pellet was then dissolved in 0.5 ml perchloric acid and kept at 100 °C for 1 h. This acid hydrolysate was neutralized using saturated KOH. Precipitates formed were removed by centrifugation and the sample was then used for HPLC analysis. Where enzymatic DNA hydrosis was employed, a similar quantity of DNA, dissolved in 500 μl 0.01 M Tris, 1 mM $MgCl_2$, pH 8.0, was incubated with 1 mg deoxyribonuclease I for 2 h, 37 °C. 0.25 units phosphodiesterase from *Crotalus durissus* venom were then added and incubation continued for 2 h. Finally 280 units alkaline phosphatase from *E. coli* were added, with a further incubation of 12 h. Enzymes were obtained from Boehringer, Mannheim.

Proteins were removed by chloroform extraction and the sample was then used for HPLC analysis.

Analysis of base content and N^6-MeA determination by HPLC. HPLC was performed using a column of 5 μm Spherisorb C8 (4.6 x 200 mm) (Phase Separations Ltd., UK). UV detection was by a variable wavelength detector (Spectromonitor III, Laboratory Data Control, USA). The solvent delivery system used was a double-reciprocating pump (Applied Chromatography Systems, UK). A 20 μl injection volume was used throughout, via a 7125 Rheodyne loop injector (Anacham, UK). For detection of N^6-MeA, the mobile phase was acetonitrile: 0.01 M phosphate buffer plus 0.01 M hexanesulphonic acid (5:95) v/v, pH 6.0. The flow rate was maintained at 1 ml/min and detection performed at 265 nm (sensitivity 0.01 AUFS). Other bases were analysed using a solvent system of 0.01 M phosphate buffer, 0.01 M hexanesulphonic acid, pH 5.0. The flow rate in these experiments was 1.5 ml/min and detection at 280 nm (sensitivity 0.05 AUFS).

Restriction endonuclease digestion and agarose gel electrophoresis. Restriction enzymes were obtained from Bethesda Research Laboratories, USA and reactions performed as recommended by the manufacturer. Digestion was performed for 6 h. Following incubation, tracking dye containing 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol was added to each sample to a final concentration of 17% (v/v). Fragments from a HindIII digestion of lambda DNA (Bethesda Research Laboratories, USA) were used as molecular weight standards. 1% agarose gels were run in 0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA, pH 8.3. Samples from Sephacryl-S1000 columns were electrophoresed for 1.5 h on a gel (0.5 cm x 20 cm x 24 cm) at 150 V. Restriction digests were analysed on gels (0.3 cm x 6.5 cm x 10 cm) and electrophoresis performed at 60 V for 2 h. Gels were stained for 30 min in ethidium bromide at 1 μg/ml and visualised with a Mineralight transilluminator (UV Light Products Ltd., USA). Gels were photographed through a Wratten no. 9 filter using a Polaroid MP-4 land camera.

Results and discussion

Determination of base content and detection of N^6-MeA in P. chrysogenum DNA

Following optimization of HPLC chromatographic and detection conditions, the method described here for determination of DNA base content was found to be simple and rapid compared to those involving radiolabelling of

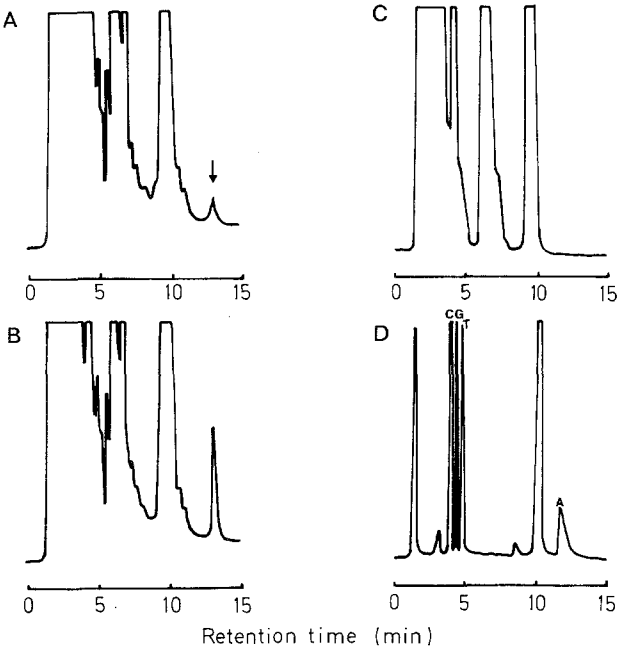

Fig. 1A–D. HPLC chromatography of DNA hydrolysates from *P. chrysogenum.* A Detection of N^6-methyladenine (↓) in strain HP36 (wild-type for 2AP sensitivity), B HP36 sample after addition of N^6-methyladenine standard, C strain HP547 (2AP sensitive), D separation of the major bases from a *P. chrysogenum* DNA hydrolysate (*G*, guanine; *C*, cytosine; *T*, thymine; *A*, adenine)

DNA, followed by thin-layer chromatography of DNA hydrolysates. Wagner and Capesius (1981), have previously described a similar technique to separate and quantitate nucleosides, including 5-methyldeoxycytidine, from plant DNA. Figure 1D shows an example whereby the major bases in *P. chrysogenum* DNA acid hydrolysates have been separated by HPLC. From calibration of external standards, the base content of *P. chrysogenum* was quantitated and the relative G+C content determined to be 51.5%. This figure correlates well with that obtained by buoyant density estimates in this laboratory (Saunders et al. 1984).

The presence of N^6-MeA in *P. chrysogenum* DNA has been detected, as demonstrated in the chromatogram in Fig. 1A. The identity of this methylated base was confirmed by an increase in peak area, when an external standard of N^6-MeA was added to the sample (Fig. 1B). Using estimates obtained for molar proportions of bases in *P. chrysogenum,* the relative amount of N^6-MeA present has been determined to be 0.1 mol percent. It has not been possible to detect 5-MeC in any DNA acid hydrolysates, although our detection limit for this base was below 28 pmoles. As 5-MeC is known to be deaminated by acid hydrolysis (Adams and Burdon 1982), enzymatic hydrolysates were also examined but no 5-MeC was detected in these samples.

DNA extracted from HP547, a 2AP, MNNG sensitive isolate, showed no N^6-MeA by HPLC analysis (Fig. 1C). As the detection limit for N^6-MeA was 1.3 pmol, it is suggested that this strain is undermethylated as compared to its progenitor strain, HP36.

Restriction endonuclease digestion of DNA from strains HP36 and HP547

It is known that the actions of certain restriction enzymes are inhibited by the presence of methylated bases in their recognition sequence and this fact has been used previously to demonstrate sequence specificity of DNA methylation (Adams and Burdon 1982). In *E. coli* the sequence 5′-G-A-T-C-3′ is methylated and therefore neither BclI nor MboI will cut this DNA (Lacks and Greenberg 1977). A comparison of restriction enzyme digests of wild type and undermethylated strains made on the basis of the general amount of degradation observed, indicated that methylation was either occurring at a different sequence to that of the Dam methylase of *E. coli* or alternatively was entirely non-specific.

Evidence for a relationship between DNA methylation and a mismatch repair like system

The results shown in Table 1 summarize the sensitivity and mutator characteristics of the strains isolated in this study. From 2,000 MNNG mutagenized colonies screened, 2 isolates were obtained which were cross-sensitive to the lethal effects of 2AP and MNNG. 2AP survival curves are shown in Fig. 2. Both HP547 and HP548 were also found to be sensitive to MMS. Although neither strains showed any altered induced mutation rates by these mutagens, HP547 was found to yield twice the number of spontaneous candidine and 5FU resistant mutants. In contrast HP548, which is not undermethylated, shows no increase in spontaneous mutation rate. From the results discussed previously and based on the model of Glickman and Radman (1980), it can be suggested that undermethylation of DNA in strain HP547 results in its inability to recognize mismatches at replication. Of seven spontaneous revertants/suppressors obtained from HP-547 which were as resistant to 2AP as wild type strains, one HP549, gave 10 times the number of spontaneous mutations. Although the degree of methylation in HP-549 has not been determined the higher spontaneous mutation frequency observed further supports the existence in *P. chrysogenum* of a DNA repair system analogous to the mismatch one in *E. coli.*

Indirect evidence has therefore been obtained in *P. chrysogenum* to allow speculation that fidelity of DNA replication could be, at least partially, monitored by a system which distinguishes methylated from unmethyl-

Table 1. Sensitivity and mutator characteristics (evidence of resistance to candidine and 5FU) of strains isolated in this study. L, Lethal effects; I, induced mutation; +, increased over parental strain; 0, same as parental strain; n.t., not tested

Strain		Strain				
		HP36	HP547 (apsA001)	HP548 (aps-002)	HP549 (apsA001, supA001)	HP550 (aps-002, sup-002)
Spontaneous		0	+ (X2)	0	+ (X10)	0
No. resistant to						
(i) candidine		0.82 ± 0.31	2.18 ± 0.27		10.1 ± (0.95)	
(ii) 5FU		0.58 ± 0.25	1.25 ± 0.34		7.5 ± (0.51)	
per 10^6 viable cells						
2AP	L	0	+	+	0	0
	I	0	0	0	0	0
MNNG	L	0	+	+	0	0
	I	0	0	0	0	0
MMS	L[a]	0	+	+	0	0
	I	n.t.	n.t.	n.t.	n.t.	n.t.

[a] Plate test only

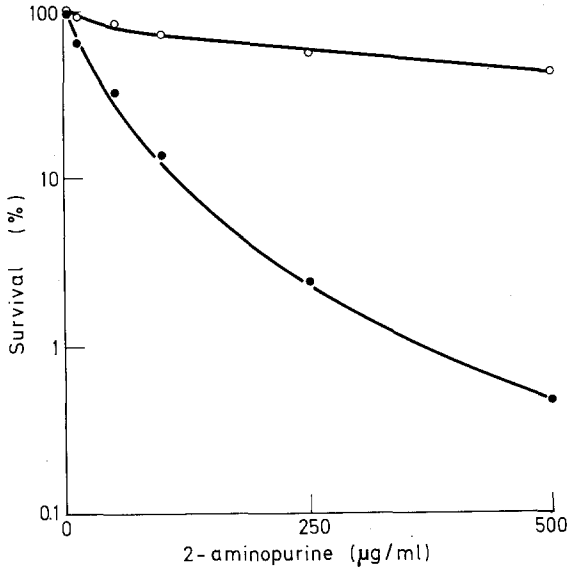

Fig. 2. Survival curves showing the lethal effects of 2-aminopurine in *P. chrysogenum* strains, wild type for 2AP sensitivity (○) and HP547 (●)

ated DNA. Analysis of the mutants obtained gave no indication that they were defective in a system which is involved in mutation fixation following excision of base analogue induced mismatches. Their defects do, however, result in an inability to repair lethal lesions caused by MMS, MNNG and the base analogue 2AP.

Acknowledgements. It is a pleasure to thank Rhône-Poulenc, Health Division, for its support and our colleagues at PCL for their useful advice and discussions.

References

Adams RLP, Burdon RH (1982) CRC Crit Rev Biochem 13:349 to 384
Ditchburn P, Giddings B, Macdonald KD (1974) J Appl Bacteriol 37:515–523
Ehrlich M, Wang RYH (1981) Science 212:1350–1357
Glickman B, Radman M (1980) Proc Natl Acad Sci USA 77:1063 to 1066
Glickman B, van den Elsan P, Radman M (1978) Mol Gen Genet 163:307–312
Hattman S, Gold E, Plotnik A (1972) Proc Natl Acad Sci USA 69:187–190
Hattman S, Kenny C, Berger L, Pratt K (1978) J Bacteriol 135: 1156–1157
Holt G, Adlard M, Lisboa E, Rogers ME, Rogers SD, Saunders G, Smith T (1984) Biochem Soc Trans 12:577–580
Lacks S, Greenberg B (1977) J Mol Biol 114:153–168
Marinus MG, Morris NR (1973) J Bacteriol 114:1143–1150
Raper KB, Alexander DF (1945) J Elisha Mitchell Sci Soc 61: 74–113
Rogers SD, Holt G (1984) Appl Microbiol Biotechnol 20:251–255
Saunders G, Rogers ME, Adlard MW, Holt G (1984) Mol Gen Genet 1984:343–345
Smith JD, Arber W, Kuhnlein U (1972) J Mol Biol 63:1–8
Tamane M, Antegueia F, Villaneuva JR, Santos T (1983) J Gen Microbiol 129:2079–2089
Wagner I, Capesius I (1981) Biochim Biophys Acta 654:52–56
Whittacker PA, Hardman N (1980) Biochem J 191:859–862

Communicated by B. S. Cox

Received October 1, 1985